

The Influence of Neural Tube-derived Factors on Differentiation of Neural Crest Cells *In Vitro*

I. Histochemical Study on the Appearance of Adrenergic Cells¹

MARTHE J. HOWARD² AND MARIANNE BRONNER-FRASER³

Department of Physiology and Biophysics, University of California, Irvine, California 92717

Abstract

The neural crest gives rise to numerous derivatives including adrenergic and cholinergic neurons, supportive cells of the nervous system, and melanocytes. In tissue culture, neural crest cells explanted from both cranial and trunk regions were found to differentiate into adrenergic neuroblasts or into pigmented cells when grown in medium containing 10% chick embryo extract. When the embryo extract concentration was lowered to 2%, no catecholamine-containing cells (as assayed by formaldehyde-induced fluorescence) were detected, although pigment cells were observed. These results suggest the presence of a factor(s) in embryo extract that promotes or supports adrenergic differentiation. To examine the possible tissue sources of this factor(s), neural tube, notochord, or somite cells were used to condition medium containing 2% embryo extract. When neural crest cells were grown in medium conditioned by neural tube cells, adrenergic neuroblasts were observed in all cultures. However, somite- and notochord conditioned medium did not support adrenergic differentiation. In addition, medium supplemented with extracts derived from central nervous system components did support adrenergic expression, whereas medium supplemented with embryo extract from which the neural tissue was removed did not. Direct contact with neural tube cell ghost membranes was unable to substitute for high embryo extract concentrations or for neural tube-conditioned medium. These results suggest that the neural tube makes a diffusible factor(s) that will support adrenergic differentiation of neural crest cells.

Neural crest cells are the precursors to most of the peripheral nervous system and a number of other structures in vertebrate embryos. Prior to migration, the neural crest appears to be a

homogeneous population of cells that originates within the dorsal margin of the neural tube. After departure from the neural tube, these cells migrate extensively to defined sites within the embryo and eventually give rise to a remarkable array of derivatives including neurons, glia, melanocytes, and neuroendocrine cells (for review, see Weston, 1970; Le Douarin, 1982). Because of this diversity in developmental potential, the neural crest has become an important system for studying both the nature and the sequence of embryonic cell lineage decisions.

The embryonic environment through which neural crest cells migrate *in vivo* appears to affect the differentiation decisions of some neural crest cells (Le Douarin and Teillet, 1974; Le Douarin et al., 1975; Noden, 1975). When regions of the neural tube containing crest cells that would normally become cholinergic neurons are transplanted into levels of the neuraxis where adrenergic cells arise, many of the transplanted cells differentiate according to their new environment and become adrenergic neurons (Le Douarin et al., 1975). Similarly, when presumptive adrenergic neural crest cells are placed in presumptive cholinergic regions, many of the transplanted cells differentiate into cholinergic neurons (Le Douarin and Teillet, 1974). These results indicate that the embryonic milieu can affect neural crest differentiation, either by induction or selection of certain phenotypes.

A successful approach to studying neural crest differentiation has been to explant neural crest cells in tissue culture under a variety of experimental conditions (Cohen and Konigsberg, 1975; Cohen, 1977; Greenberg and Schrier, 1977; Newgreen et al., 1979; Smith et al., 1979; Kahn et al., 1980; Fauquet et al., 1981; Loring et al., 1982). Tissue culture provides a somewhat defined environment in which one can examine the effects of experimental alterations on neural crest cell differentiation. Under appropriate culture conditions, neural crest cells give rise to pigmented cells and neurons containing catecholamines, acetylcholine, serotonin, or somatostatin (Cohen, 1977; Greenberg and Schrier, 1977; Kahn et al., 1980; Sieber-Blum et al., 1981; Maxwell et al., 1982). The tissue culture environment appears to influence phenotypic expression in neural crest cells; e.g., fibronectin substrates *in vitro* increase the percentage of adrenergic derivatives arising in neural crest cultures (Sieber-Blum et al., 1980; Loring et al., 1982). Thus, some molecules produced by embryonic tissues surrounding neural crest pathways may alter neural crest differentiation by promoting, restricting, or selecting for certain avenues of differentiation.

Previous investigators have examined the role of tissue interactions in neural crest differentiation. Cohen (1972) co-cultured neural crest explants together with other embryonic tissues on the chick chorioallantoic membrane. Only in the presence of somite cells and the ventral neural tube did adrenergic differentiation occur. These

Received February 27, 1985; Revised April 22, 1985;
Accepted April 22, 1985

¹ We thank Drs. Scott Fraser and James Coulombe for helpful comments on the manuscript, and Georgia Guillory for her excellent technical assistance. This work was partially supported by United States Public Health Service Grant 15527-01 and a Basic Research grant for the March of Dimes Birth Defects Foundation.

² Present address: Division of Pharmacology, M-013-H U.C.S.D., La Jolla, CA. 92193.

³ To whom reprint requests should be addressed at her present address: Department of Medicine, Developmental Biology Center, University of California, Irvine, Irvine, CA. 92717.

studies were extended by Norr (1973), who demonstrated that contact with somite cells was necessary for adrenergic expression, but that the ventral neural tube could exert its effect across a Millipore filter, suggesting that the neural tube was producing some diffusible substance. Cohen (1977) subsequently showed that neural crest cells could undergo autonomous differentiation into catecholamine-containing cells when grown in culture medium containing horse serum and high concentrations of embryo extract.

In a related study using different culture conditions, Fauquet et al. (1981) examined neural crest differentiation and were unable to detect autonomous expression of adrenergic neuroblasts. Two major differences exist in the growth conditions between their studies and those of Cohen (1977): (1) the neural crest explants of Fauquet et al. (1981) consisted of neural folds alone, which presumably contain no neural tube elements, whereas those of Cohen (1977) were obtained from neural tubes from which neural crest cells subsequently migrated; therefore, the latter explants contained neural tube cells for the first 24 to 48 hr in culture; (2) the medium used by Fauquet et al. (1981) contained only 2% chick embryo extract whereas that of Cohen (1977) contained 10% embryo extract. Taken together, these results suggest that either co-culture with neural tube cells and/or high concentrations of embryo extract are required for neural crest cells to express adrenergic traits.

The present investigation extends these previous studies to examine the effects of different embryonic tissues and culture conditions on adrenergic differentiation of cranial and trunk neural crest cells *in vitro*. In particular, we have examined the requirements for co-culture with neural tube cells for the expression of adrenergic traits. The results resolve the differences between the studies of Fauquet et al. (1981) and Cohen (1977). Preliminary reports on some aspects of this work have appeared elsewhere (Howard et al., 1982; Howard and Bronner-Fraser, 1983).

Materials and Methods

Neural crest cell cultures

Japanese quail (*Coturnix coturnix japonica*) embryos were used for all experiments. The embryos were incubated at 38°C in a forced air incubator until they reached the appropriate stages of development. After removal of the neural tube, neural folds, or migrating neural crest cells from the embryos, the explants were washed in Howard's Ringer's solution and plated onto 35-mm Petri dishes coated with human fibronectin (25 µg/ml) or rat-tail collagen (1 mg/ml). Comparable results were obtained on both substrates. For one set of experiments, neural crest cells were plated onto neural tube cell ghosts. Two to seven explants were placed onto each plate and were allowed to attach to the dish for several hours, at which time culture medium was added. After 24 hr, the original explants and any non-neural crest cells were carefully scraped from the dish using an electrolytically sharpened tungsten needle, leaving only those neural crest cells that had migrated away from the initial explant. The culture medium was removed and replaced with fresh medium. All plates were screened on the following 2 days for the presence of motoneurons which would indicate that some residual neural tube cells were present in the culture (Fauquet et al., 1981). Motoneurons can be identified by their long axons, since they are the only cell type emerging from the explanted neural tubes that elicit processes within 2 days after explantation. Those plates containing motoneurons were discarded. Cultures were fed fresh medium every other day.

Cranial crest cell cultures. Cranial neural crest cells were obtained from stage 8+ to stage 9- embryos (according to the criteria of Hamburger and Hamilton, 1951). At these stages, the neural folds had begun to fuse at the level of the mesencephalon from which the explants were taken. The endoderm, metencephalon, and rhombencephalon were dissected away from the mesencephalic folds using fine glass needles. The mesencephalic tissue contained the neural crest cells within the fused mesencephalic neural folds, as well as a small portion of lateral plate mesenchyme, and notochord. The tissue was split lengthwise down the midline, the notochord was removed, and the remaining tissue was washed and plated.

Cranial neural fold cultures. The cranial neural folds were dissected from stage 8- embryos in which the folds were not yet fused. The neural crest cells at this stage are situated within the neural folds between ectoderm and the presumptive neural tube. After the rhombencephalon and metencephalon

were dissected away, the dorsal portion of the mesencephalic neural fold was removed with fine glass needles by making a series of cuts between the ectoderm and the neural groove. The neural folds were washed in Howard's Ringer's solution before plating.

Migrating cranial crest. Mesencephalic neural crest cells that were in the process of migrating were prepared according to the procedure of Fauquet et al. (1981). Embryos at stages 9+ to 10+ of development were used for these dissections. At these stages, many neural crest cells have migrated from the dorsal neural tube and are situated in the lateral plate mesenchyme just under the ectoderm and adjacent to the presumptive otic vesicle. Neural crest cells that had migrated into the lateral plate mesenchyme were obtained by dissecting free a portion of the mesenchyme just rostral to the initial site of emigration at the mesencephalon. Using fine glass needles, the lateral plate mesenchyme (containing migrating neural crest cells) was separated from the neural tube, washed, and plated.

Trunk neural crest cultures. Neural crest cultures from the trunk region were prepared according to the method of Cohen (1977). The neural tubes plus surrounding tissue in the posterior regions of stage 13 to 14 embryos were removed using a tungsten needle. For most experiments, only the closed portion of the neural tube was used; in a few cases, both closed and unclosed regions of the neural tube were included in the dissection. The neural tubes were then separated from surrounding tissue by treatment with 0.1% crude trypsin (Worthington) for 20 min at 4°C. The reaction was stopped by adding tissue culture medium containing endogenous trypsin inhibitors. Neural tubes were released from other tissues by gentle trituration and subsequently were placed onto culture dishes.

Trunk neural fold cultures. Neural folds from the trunk region were obtained from stage 12+ to 13+ embryos. Using a tungsten needle, the embryos were bisected at the level of somite 17. The forming neural tube (containing the neural folds) just posterior to the last somite and anterior to Hensen's node were removed with glass dissecting needles, and the dorsalmost portion of the neural folds was separated from the presumptive neural tube. After washing in Howard's Ringer's solution, the trunk neural fold explants were placed in culture.

Tissue culture growth media

The growth media used to feed neural crest cultures consisted of Eagle's minimum essential medium (MEM) (Grand Island Biological Co., GIBCO) supplemented with 15% horse serum (GIBCO) and 11-day whole chick embryo extract. In addition, extracts derived from a variety of tissue sources were prepared at concentrations of 2%, 5%, or 10%.

Preparation of embryo extract

Whole chick embryo extract (EE). Embryo extract was prepared from White Leghorn chicken embryos incubated at 38°C for 11 days. The eggs were candled and washed with 70% ethanol. After removal of the embryos from the shell, the beaks and eyes were removed and discarded. The embryos were washed with sterile MEM, minced, and macerated by pushing through a 60-ml syringe. An equal volume of MEM was added to the crude extract, which was then stirred at 4°C for 45 min. Sterile hyaluronidase (1 mg/25 gm of tissue) was added to reduce the viscosity of the EE, and the mixture was stirred for an additional 15 min. The extract was sedimented at 31,000 × *g* for 30 min at 4°C, the pellet was discarded, and the supernatant was sedimented at 95,000 × *g* for 60 min at 4°C. The supernatant was collected, filter-sterilized, and used immediately as a component of complete growth medium which was stored at -80°C.

Embryo extract lacking central nervous system tissues (NNE). NNE was prepared as described above for EE with one modification. Following removal of the beak and eyes, the brain and spinal cord were removed and retained in MEM on ice. The remainder of the embryo was used to prepare an extract as described above.

Brain and spinal cord extract. The brains and spinal cords were removed from 11-day chicken embryos and a neural tube extract (NTE) was prepared as described above.

Preparation of conditioned media (CM)

Cultures of neural tubes, notochords, or somites were prepared as described above for trunk neural crest cell cultures. A block of tissue from the trunk region containing the neural tube, notochord, and somites was separated from the rest of the embryo using a tungsten needle. After trypsinization, either the neural tubes, notochords, or somites were separated from surrounding tissues by gentle trituration, washed in Howard's Ringer's solution, and collected. Neural tubes, notochords, or somites were separately plated onto culture dishes and were fed growth medium containing 2% EE.

After 2 days, the conditioned medium (CM) was collected, filtered-sterilized, and used to feed neural crest cell cultures.

Preparation of neural tube cell membrane ghosts

Neural tubes were prepared and plated as described above. After several days in culture, the cells were lysed by adding sterile distilled water. After lysis, a layer of cell membranes visible under the phase contrast microscope remained on the culture dish. The membrane ghosts were washed with distilled water and used as substrates onto which neural crest cells were plated.

Histochemical detection of catecholamines

Catecholamine-containing cells were identified by formaldehyde-induced fluorescence (Falck et al., 1962). Upon heating in the absence of water, catecholamines condense with formaldehyde vapors to form a fluorescent product with a characteristic excitation/emission of 420/480 nm, respectively. For analysis of catecholamines in neural crest cell cultures, the culture medium was removed, and the cultures were air dried and exposed to formaldehyde vapors at 80°C for 1 hr. The cultures were observed under a Zeiss Universal microscope equipped for epifluorescence, using a BP 405/6 excitation filter and an LP 435 barrier filter.

Results

These experiments were designed to address some aspects of neural crest cell lineage decisions. In particular, we have looked for the presence of factors in growth medium or synthesized by embryonic tissues that influence neural crest differentiation into adrenergic cells. For the purpose of this study, we define an adrenergic cell as one containing catecholamine stores that are identifiable by means of formaldehyde-induced fluorescence; catecholamines fluoresce with a characteristic blue/green color after reaction with formaldehyde vapors and, therefore, are easily identified with epifluorescence microscopy.

Effects of EE on neural crest cell differentiation

Some neural crest cells will differentiate into adrenergic cells when grown in medium supplemented with 10% EE and 15% horse serum (Cohen, 1977), but not when grown under different culture conditions with reduced concentrations of EE (Fauquet et al., 1981). Here, we examine the effects of EE concentration on the differentiation of both cranial and trunk neural crest cells into adrenergic cells. Cultures were grown in media containing MEM, 15% horse serum, and EEs added at concentrations of 10%, 5%, or 2%. The 2% EE was chosen because this concentration was used to supplement medium in the experiments of Fauquet et al. (1981). Although Fauquet et al. (1981) supplemented their medium with 15% fetal calf serum, we used horse serum for these experiments because survival of neural

crest cells was markedly longer in medium supplemented with horse serum (Howard, 1984), although the same range of derivatives was observed with both sera.

Neural crest explants were prepared in two ways: (1) from neural folds, or (2) from explanted neural tubes. The former presumably do not contain neural tube cells, whereas the latter cultures contain some neural tube cells during the first 24 hr in culture, after which time the neural tube cells were scraped away.

Cranial neural crest cells. Cranial crest cultures were maintained for 6 to 62 days. In all cranial crest cultures grown in either 10% or 5% EE, many cells differentiated into adrenergic cells, as assayed by formaldehyde-induced fluorescence, and pigmented cells, as identified by melanin granules (Table I). In contrast, cranial crest cultures grown in medium supplemented with 2% EE did not contain adrenergic cells as assayed by formaldehyde-induced fluorescence (Table I). The cultures were apparently healthy since pigment cells were observed in all cultures and began to differentiate between day 5 and 6 *in vitro*. Thus, this experiment suggests that cranial neural crest cells require some component(s) of the embryo extract in a concentration greater than 2% in order to express the adrenergic phenotype.

Trunk neural crest cultures. Trunk neural crest cultures were grown in the presence of 10%, 5%, and 2% EE. After 6 or more days, the cultures were examined for the presence of pigment cells and catecholamine-containing cells. In cultures grown in 10% or 5% EE, both adrenergic cells and pigment cells were observed (Fig. 1a, Table I). When the growth medium was supplemented with only 2% EE, melanocytes were present in all cultures but no adrenergic differentiation occurred. These observations are analogous to those found for cranial neural crest cultures. Thus, in low concentrations of embryo extract, the tissue culture conditions are permissive for melanogenesis but restrictive for adrenergic differentiation of both trunk and cranial neural crest cells. This suggests that 11-day chicken embryo extract may contain some factor or factors that are required for the expression of adrenergic cells.

The effects of neural tube on neural crest cell differentiation

In the previous series of experiments, both trunk and cranial crest cells were explanted with a portion of the neural tube which remained in the culture dish for the first 24 hr. In order to separate the effects of the neural tube on adrenergic differentiation from EE effects, cultures of neural crest cells lacking the neural tube were prepared. These explants were either prepared from tips of neural folds which contain neural crest cells prior to their migration, or were made from the lateral mesenchyme which contains migrating neural crest cells that had already left the neural tube.

TABLE I
Differentiation of trunk and cranial neural crest cells

Neural crest cells from cranial or trunk regions were grown in different concentrations of whole chick EE. Explants were either derived from the neural folds shortly before neural tube closure, or from the neural tube after closure. The cultures were analyzed for catecholamine histofluorescence and for the presence of pigment cells.

Dissection	Presence of Neural Tube in Initial Explant	Catecholamine Histofluorescence (% EE)			Pigment Cells (2 to 10% EE)	Days
		2	5	10		
Cranial crest	yes	0/68 ^a (0/9) ^b	12/12 (5/5)	55/55 (15/15)	125/125 (29/29)	6-62
Trunk crest	yes	0/37 (0/10)	20/20 (9/9)	68/68 (18/18)	125/125 (37/37)	7-16
Cranial neural fold	no	0/20 (0/8)	ND ^c	27/27 (8/8)	47/47 (16/16)	7
Migrating mesencephalic crest	no	0/25 (0/6)	10/10 (3/3)	25/25 (6/6)	125/125 (15/15)	7-21
Trunk neural fold	no	0/22 (0/8)	11/11 (4/4)	25/25 (7/7)	58/58 (19/19)	6-21

^a Number of explants positive per total explants.

^b Number of plates positive per total plates.

^c ND, not determined.

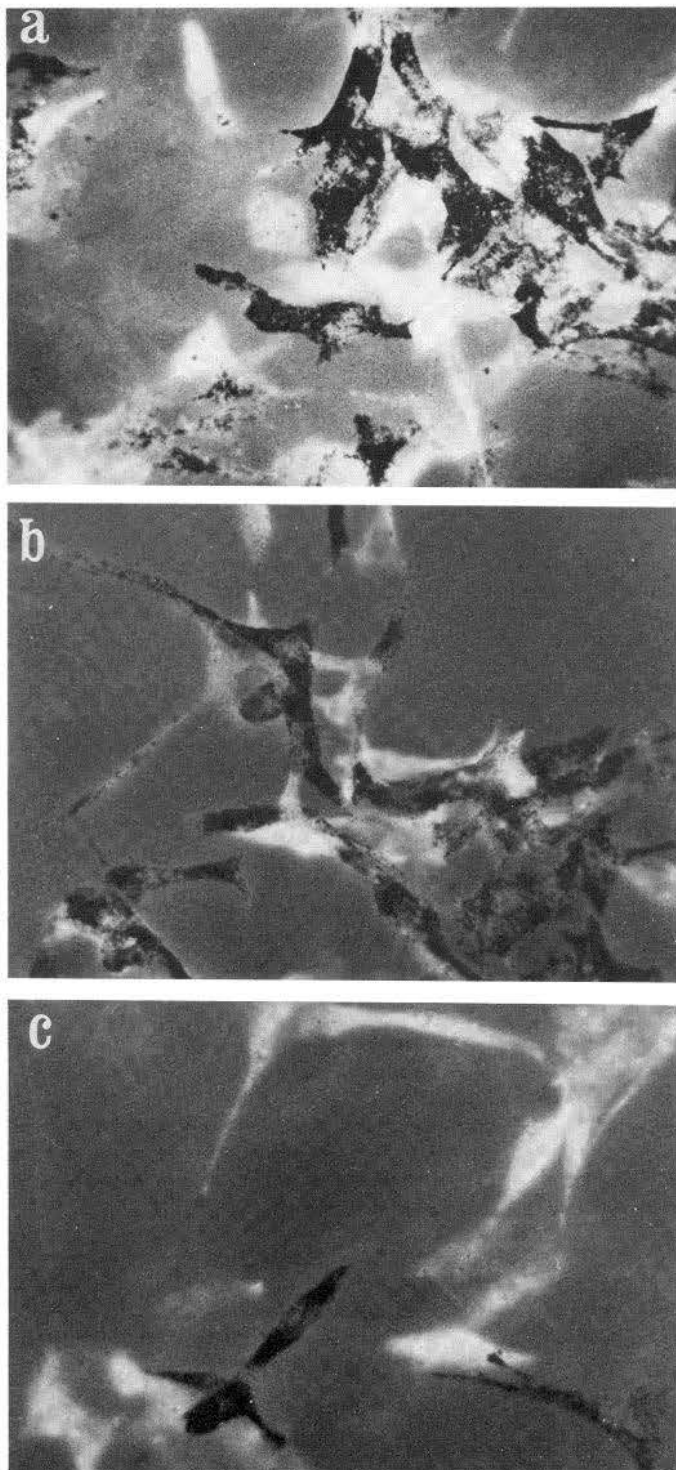


Figure 1. Fluorescence superimposed on brightfield photomicrographs of catecholamine histofluorescence in neural crest cells derived from neural fold explants. *a*, Cells were grown in medium containing 10% EE. Note the numerous fluorescent cell bodies indicating the presence of adrenergic cells in these cultures. Numerous pigment cells, identified by the dark melanin granules within their cytoplasm, were also present. *b*, Cells were grown in medium containing 10% NTE. Both catecholamine-containing cells and pigment cells differentiated under these conditions. *c*, Cells were grown in neural tube CM which contained 2% EE. Both adrenergic and pigmented cells were observed under these conditions.

Cranial region. In the cranial region, two types of explants were prepared that do not contain any portion of the mesencephalic neural tube: (1) cranial neural folds, and (2) migrating mesencephalic neural crest cells. In experiments parallel to those reported above,

cranial neural fold or migrating mesencephalic crest cells were grown in medium containing either 10%, 5%, or 2% EE. Regardless of the EE concentration, melanocytes differentiated in both types of cultures. Adrenergic differentiation, however, occurred only when the medium contained 5% or 10% EE (Table I) for both cranial neural fold cultures and migrating mesencephalic crest cultures. When the concentration of EE was lowered to 2%, no adrenergic cells were detected. These results are analogous to those reported above for cranial neural crest cultures (containing the mesencephalic neural tube for the first 24 hr in culture) and, therefore, suggest that the initial presence of the neural tube is not necessary for the expression of adrenergic and melanotic derivatives. Thus, embryo extract concentrations of 5% and 10% appear to be sufficient for both adrenergic and melanotic expression of mesencephalic neural crest cells with or without the initial presence of the neural tube.

Trunk region. Trunk neural fold explants that lacked associated neural tube cells were prepared from the posterior neural folds prior to neural crest migration. Similar to the results observed with cranial neural crest cells, the trunk neural fold cells gave rise to both pigmented and adrenergic cells in the presence of 5% or 10% EE. When the EE concentration was lowered to 2%, no catecholamine-containing cells were detected although pigment cells did differentiate (Table I). Thus, for all dissections of cranial and trunk neural crest, regardless of the initial presence or absence of neural tube cells, medium containing 5% or 10% EE resulted in adrenergic differentiation whereas medium containing 2% EE was unable to support adrenergic differentiation.

The effects of extracts derived from different embryonic tissues on adrenergic differentiation

EE lacking central nervous system (CNS) elements. Extract was prepared from 11-day chicken embryos from which the brain and spinal cord were removed. This extract lacking CNS components (NNE) was then used to supplement growth medium at concentrations of 2% or 10%. Several types of cultures (either initially containing or lacking neural tube cells) were grown under these conditions. The results are summarized in Table II. In either 2% or 10% NNE medium, pigment cells differentiated, but no adrenergic cells were observed. Because 10% whole EE is sufficient to promote adrenergic differentiation in neural crest cultures yet 10% NNE cannot, these results suggest that the factors in whole EE that appear to be important for adrenergic differentiation are either lacking or are present in subthreshold concentrations in NNE.

Brain and spinal cord extract (NTE). In order to determine whether the CNS produces factors which support adrenergic differentiation of some neural crest cells, extract was made from the brain and spinal cord of 11-day chick embryos. This NTE was used to supplement growth medium at concentrations of 2% or 10% which was then fed to cranial and trunk neural crest cultures. In both 10% and 2% NTE, adrenergic cells were observed between 7 and 10 days in all neural crest cultures, including those lacking neural tube cells in the initial explant (Fig. 1*b*). Pigmented cells were observed in all cultures as well. These results are summarized in Table III. Thus, the brain and spinal cord appear to synthesize some substance or substances that will support differentiation of adrenergic cells from undifferentiated neural crest cells.

CM effects on neural crest differentiation

The experiments described above suggest that some factor(s), which may be produced by CNS elements, is required for adrenergic differentiation of some neural crest cells *in vitro*. Therefore, we examined whether tissues encountered by migrating neural crest cells could produce diffusible substances which affect adrenergic differentiation by conducting a series of CM experiments. Neural tube, somite, and notochord cells were used to condition growth medium containing 2% EE; medium containing 2% EE cannot, on its own, support adrenergic differentiation (see Table I). The CM was then fed to neural crest explants from various axial levels and derived

TABLE II
Differentiation of trunk and cranial neural crest cells in NNE

Neural crest cells from cranial or trunk regions were grown in two different concentrations of extract prepared from embryos lacking central nervous system elements (NNE) and were assayed for catecholamine histofluorescence and the presence of pigment cells.

Dissection	Presence of Neural Tube in Initial Explant	NNE (%)	Catecholamine Histofluorescence	Pigment Cells	Days
Cranial crest	yes	10	0/16 ^a (0/7) ^b	16/16 (7/7)	7-10
		2	0/17 (0/7)	17/17 (7/7)	10
Cranial neural fold	no	10	ND ^c	ND	ND
		2	0/16 (0/6)	16/16 (6/6)	7-10
Migrating mesencephalic crest	no	10	0/17 (0/7)	17/17 (7/7)	7
		2	0/11 (0/4)	11/11 (4/4)	7
Trunk crest	yes	10	0/7 (0/3)	7/7 (3/3)	7-10
		2	0/12 (0/4)	12/12 (4/4)	7-10

^a Number of explants positive per total explants.

^b Number of plates positive per total plates.

^c ND, not determined.

TABLE III
Differentiation of trunk and cranial neural crest cells in NTE

Neural crest cells from cranial or trunk regions were grown in two different concentrations of extract prepared from the brain and spinal cord (NTE). Cultures were analyzed for catecholamine histofluorescence and the presence of pigment cells.

Dissection	Presence of Neural Tube in Initial Explant	NTE (%)	Catecholamine Histofluorescence	Pigment Cells	Days
Cranial crest	yes	10	16/16 ^a (5/5) ^b	16/16 (5/5)	7-10
		2	19/19 (7/7)	19/19 (7/7)	10
Cranial neural fold	no	10	30/30 (9/9)	30/30 (9/9)	7-10
		2	26/26 (10/10)	26/26 (10/10)	7-8
Migrating mesencephalic crest	no	10	4/4 (1/1)	4/4 (1/1)	7
		2	3/3 (1/1)	3/3 (1/1)	10
Trunk crest	yes	10	11/11 (5/5)	11/11 (5/5)	7-9
		2	17/17 (8/8)	17/17 (8/8)	7

^a Number of explants positive per total explants.

^b Number of plates positive per total plates.

TABLE IV
Differentiation of trunk and cranial neural crest cells in CM

Cranial or trunk neural crest cells were grown in medium containing 2% EE that had been conditioned by neural tube, somite, or notochord cells. Cultures were assayed for catecholamine histofluorescence and for pigment cells.

Dissection	Presence of Neural Tube in Initial Explant	Conditioning Tissue	Catecholamine Histofluorescence	Pigment Cells	Days
Cranial crest	yes	Neural tube	6/6 ^a (3/3) ^b	6/6 (3/3)	12
		Somite	0/14 (0/5)	14/14 (5/5)	12
		Notochord	0/9 (0/3)	9/9 (3/3)	9-12
Cranial neural fold	no	Neural tube	29/29 (9/9)	29/29 (9/9)	9-12
		Somite	0/9 (0/3)	9/9 (3/3)	7-9
		Notochord	0/9 (0/3)	3/9 (1/3)	12
Trunk neural fold	no	Notochord	0/8 (0/4)	3/8 (1/4)	12

^a Number of explants positive per total explants.

^b Number of plates positive per total plates.

from explants of either neural folds or neural tubes. With the exception of some cultures fed notochord CM, pigment cells were observed in most cultures regardless of the conditioned medium used.

In all cultures examined, neural tube CM was able to support adrenergic expression of neural crest cells (Figure 1c, Table IV). In contrast, no catecholamine-containing cells were detected in cultures grown in medium conditioned by somite or notochord cells. These results indicate that the neural tube but not notochord or somite cells synthesizes and secretes some factor or factors that will support expression of adrenergic traits from neural crest precursors.

Although the neural tube appears to synthesize some substances which promote adrenergic differentiation, the presence of the neural tube for the first 24 hr was not sufficient for adrenergic expression in neural crest cultures grown in 2% EE containing medium. We therefore examined whether continuous co-culture of neural crest cells with neural tube cells would result in adrenergic differentiation regardless of the EE concentration. Cranial neural crest cells were grown in medium containing 2% EE, and the neural tube cells from the original explants, that would normally be scraped away after 24 hr, were left in the culture dish. As predicted by the data above, in 33 of 33 explants (8 of 8 plates) examined, both adrenergic and

pigmented cells were observed when neural tube cells remained in the culture dish for extended periods of time.

Effects of neural tube cell membrane ghosts

Neural crest cells were grown on neural tube cell membrane ghosts in order to determine whether continuous contact with neural tube membranes could also promote differentiation of adrenergic neurons. Neural crest cells were plated onto ghosts of neural tube cells (see "Materials and Methods") in medium containing 2% or 10% EE. Regardless of the EE concentration, pigment cells differentiated in these cultures. However, adrenergic cells were only observed on the membrane ghosts in the presence of medium containing 10% EE. These results (summarized in Table V) indicate that contact with neural tube membrane ghosts is not sufficient to cause catecholaminergic differentiation of neural crest cells and support the results described above indicating that the neural tube produces and releases a diffusible factor that promotes adrenergic expression.

Discussion

The results presented in this paper show that neural crest cell differentiation into adrenergic cells requires diffusible factors synthesized by other embryonic tissues. In both trunk and cranial neural crest cultures, some cells differentiated into catecholamine-containing cells when grown in the presence of high concentrations of EE (5% or greater); however, adrenergic differentiation did not take place when the EE concentration was lowered to 2%. Embryo extract, therefore, contains some factors that support adrenergic expression. Identical data were obtained for neural crest cells grown in the absence of neural tube cells as for those co-cultured with neural tube cells for the first 24 hr, indicating that the initial presence of the neural tube does not alter the results. Extracts prepared from neural tissue alone were able to support adrenergic differentiation, whereas those prepared from embryos lacking nervous tissue did not. This suggests that the factors are of neural tissue origin. Neural crest cells grown in neural tube CM also differentiated into adrenergic cells, indicating that the factors may be diffusible. Cells grown in medium conditioned by somite or notochord cells did not produce adrenergic cells; thus, the factors that promote adrenergic differentiation of neural crest cells appear to be either absent in somite and notochord tissues, or are not produced in sufficiently high concentrations to be effective.

It had previously been suggested that neural crest cells require the presence of other embryonic tissues in order to differentiate into adrenergic neurons (Cohen, 1972; Norr, 1973). These investigators found that adrenergic differentiation of neural crest cells grown on the chorioallantoic membrane or grown on Millipore filters was dependent upon the presence of somites or the ventral neural tube. The neural tube was effective in a *trans*-filter configuration to the neural crest cells (Norr, 1973), suggesting that this tissue may

produce some diffusible substance that supports adrenergic differentiation. In contrast, somite cells were only effective when *cis*-filtered to the neural crest cells. Both somite and neural tube cells synthesize fibronectin, which is known to enhance adrenergic differentiation of neural crest cells *in vitro* (Sieber-Blum et al., 1981). Therefore, the effects of somite cells on adrenergic differentiation may be mediated by fibronectin. However, fibronectin is primarily found on the dorsal neural tube and little is detectable by immunocytochemistry on the ventral tube surface (our unpublished observation), indicating that the effect of the ventral neural tube may be mediated by another factor.

In intact embryos, removal of the neural tube and notochord following the migration of some neural crest cells results in absence of dorsal root and sympathetic ganglia (Teillet and Le Douarin, 1983). This indicates that neural crest cells cannot differentiate properly into these ganglia without the neural tube and notochord. *In vitro*, however, neural crest cells that are grown in a rich culture medium apparently can form adrenergic derivatives in the absence of other tissues (Cohen, 1977). These neural crest explants were co-cultured with neural tube cells for the first 24 to 48 hr. Using lower concentrations of EE and neural crest explants which contained no neural tube cells, Fauquet et al. (1981) were unable to detect catecholamines in neural crest cultures. They concluded that initial co-culture with neural tube cells was necessary for the differentiation of adrenergic cells. These differences between the studies of Cohen (1977) and Fauquet et al. (1981) suggest that adrenergic differentiation may require: (1) the presence of neural tube cells in culture for 24 to 48 hr, or (2) a growth medium containing more than 2% EE.

Our study demonstrates that neural crest cells can differentiate into adrenergic cells either in the initial presence or absence of the neural tube. We find that EE concentrations of 5% or 10% were able to support adrenergic expression of cells from explants that initially contained the neural tube, as well as from explants that contain no neural tube cells. If neural crest cells are continually co-cultured with neural tube cells or fed neural tube CM, an EE concentration of 2% is sufficient to result in adrenergic differentiation. Therefore, there appears to be some factor or factors present in EE and neural tube CM that supports adrenergic differentiation of some neural crest cells. For cultures grown in 2% EE medium, adrenergic cells failed to differentiate if the neural tube was removed after 24 hr. Thus, the presence of the neural tube for the first day in culture was not sufficient to support differentiation. These data reconcile the discrepancies between the experiments of Cohen (1977) and Fauquet et al. (1981), and suggest that the support of adrenergic differentiation provided by 10% EE can also be provided by diffusible substances produced by the neural tube.

The present study used five different neural crest explants taken from different levels along the neural axis and from neural crest cells at various stages of migration in order to explore regional variations in differentiation potential and to provide reproducibility. Neural crest cells taken from all levels of the neural axis gave similar results with respect to the extent or the nature of the identifiable crest derivatives formed; some cells within both cranial and trunk neural crest cultures gave rise to pigmented cells or adrenergic cells. In the intact embryo, derivatives arising from trunk neural crest are quite different from those derived from the cranial neural crest. For example, cranial neural crest cells do not normally give rise to pigment or adrenergic cells *in vivo* (Weston, 1970).

Pigment cells differentiated under all of the growth media conditions utilized in this study. This suggests that the factors necessary for the differentiation of melanocytes were present in all of the various media used, even though the factors necessary for adrenergic expression were sometimes missing. Derby and Newgreen (1982) have described a high molecular weight factor present in EE and in some lots of fetal calf serum that is required for pigment cell differentiation; pigmentation does not occur when molecules in its molecular weight range are removed by dialysis. These investigators showed that this factor can be synthesized by neural tube cells,

TABLE V

The effects of contact with neural tube cell ghosts on differentiation of neural crest cells

Cranial neural crest cells were grown on neural tube cell membrane ghosts in medium containing either 2% or 10% EE. Cultures were assayed for catecholamine histofluorescence and for pigment cells.

Dissection	Presence of Neural Tube in Initial Explant	Catecholamine Histofluorescence (% EE)		Pigment Cells (2 to 10% EE)	Days
		2	10		
Cranial crest	yes	0/8 ^a	8/8	16/16	9
		(0/4) ^b	(4/4)	(8/8)	
Migrating mesencephalic crest	no	0/8	8/8	16/16	9
		(0/4)	(4/4)	(8/8)	

^a Number of explants positive per total explants.

^b Number of plates positive per total plates.

since the neural tube can also stimulate melanogenesis in the absence of embryo extract. Our results confirm the results of these previous investigators in suggesting that this pigment-promoting factor is present in embryo extract concentrations as low as 2%. Evidence suggesting that the factors promoting melanogenesis and adrenergic differentiation are distinct includes: (1) when pigmentation is inhibited by removing high molecular weight molecules, neurogenesis still occurs (Derby and Newgreen, 1982); and (2) the factors are in different molecular weight ranges, since the adrenergic promoting factor appears to have a low molecular weight (M. J. Howard, and M. Bronner-Fraser, submitted for publication). It should be noted that a limited number of neural crest derivatives have been detected in tissue culture. This may indicate that an appropriate environment for the expression of some other derivatives has not yet been found. Still other derivatives have not yet been identified *in vitro* due to the lack of appropriate phenotypic markers.

Other factors have been described that influence the phenotypic expression of neural crest cells and their derivatives. Sympathetic neurons, derived from trunk neural crest cells, normally synthesize catecholamines. When explanted in tissue culture and grown in the presence of non-neural cells, medium conditioned by non-neural cells, or chick EE, the sympathetic ganglion cells stop making catecholamines and begin synthesizing acetylcholine (Patterson and Chun, 1974, 1977a, b; Johnson et al., 1976; Ross et al., 1977; Iacovitti et al., 1981). This adrenergic to cholinergic transition has been observed with single neurons in tissue culture (Potter et al., 1981). A glycoprotein in the molecular weight range of 40,000 to 45,000 appears to be the molecule responsible for this transition. Factors present in heart CM can affect the differentiation of neural crest cells, by promoting cholinergic differentiation while suppressing catecholamine content and melanin production (Sieber-Blum and Kahn, 1982). Some adrenergic traits can also be enhanced in cholinergic cells by co-culture with the notochord (Teitelman et al., 1985). In addition to diffusible factors, substrate-bound material can influence neural crest differentiation. For example, fibronectin or somite conditioned substrates tend to suppress melanogenesis while promoting adrenergic differentiation (Sieber-Blum et al., 1981; Loring et al., 1982). In addition, substrate-bound factors can influence neurotransmitter expression in cultures of sympathetic neurons (Hawrot, 1980).

The results of the present study demonstrate that diffusible factors exist that are able to promote adrenergic differentiation. The neural tube is one tissue that apparently synthesizes this factor and secretes it into the extracellular spaces. Whole EE and EE made from neural tissue also contain this factor. In the studies of Cohen (1972) and Norr (1973), neural crest cells gave rise to adrenergic derivatives when grown in the presence of neural tube and somite cells. Our findings suggest that support of adrenergic expression by the neural tube may be the result of a diffusible factor. Because this factor is also present in EE, neural crest cells can differentiate into adrenergic neurons in the absence of other tissue when the EE concentrations are sufficiently high (Cohen, 1977). Although our studies have examined one factor that supports adrenergic expression, it is unlikely that one factor alone can account for the complex decisions involved in the differentiation process. Rather, a multitude of factors is likely to act in concert to influence phenotypic expression of an individual cell. To understand better the role of the factor synthesized by neural tube cells in cell lineage decisions, we have undertaken a biochemical investigation of the neurotransmitter biosynthetic enzymes dopamine β -hydroxylase and choline acetyltransferase. The results suggest that the neural tube-derived factor increases activity of the adrenergic enzyme in some neural crest cells, while simultaneously suppressing or delaying the activity of the cholinergic enzyme. These findings are described in detail elsewhere (M. J. Howard, and M. Bronner-Fraser, submitted for publication).

References

- Cohen, A. M. (1972) Factors directing the expression of sympathetic nerve traits in cells of neural crest origin. *J. Exp. Zool.* 179: 167-182.
- Cohen, A. M. (1977) Independent expression of the adrenergic phenotype by neural crest cells *in vitro*. *Proc. Natl. Acad. U. S. A.* 74: 2899-2903.
- Cohen, A. M., and I. R. Konigsberg (1975) A clonal approach to the problem of neural crest determination. *Dev. Biol.* 46: 262-280.
- Derby, M. A., and D. F. Newgreen (1982) Differentiation of avian neural crest cells *in vitro*: Absence of a developmental bias toward melanogenesis. *Cell Tissue Res.* 225: 365-378.
- Falck, B., N. A. Hillarp, G. Thieme, and A. Torp (1962) Fluorescence of catecholamines and related compounds with formaldehyde. *J. Histochem. Cytochem.* 10: 348-354.
- Fauquet, M., J. Smith, C. Ziller, and N. M. Le Douarin (1981) Acetylcholine and catecholamine synthesis in neural crest cells cultured *in vitro*. *J. Neurosci.* 1: 478-492.
- Greenberg, J. H., and B. K. Schrier (1977) Development of choline acetyltransferase activity in chick cranial neural crest cells in culture. *Dev. Biol.* 61: 86-93.
- Hamburger, V., and H. L. Hamilton (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* 88: 49-92.
- Hawrot, E. (1980) Cultured sympathetic neurons: Effects of cell-derived and synthetic substrata on survival and development. *Dev. Biol.* 74: 136-151.
- Howard, M. J. (1984) Environmental factors affecting the adrenergic differentiation of quail neural crest cells. Ph.D. dissertation, University of California, Irvine.
- Howard, M. J., L. Tomosky-Sykes, and M. Bronner-Fraser (1982) Adrenergic differentiation of neural crest cells *in vitro* without the neural tube. *Soc. Neurosci. Abstr.* 8: 257a.
- Howard, M. J., and M. Bronner-Fraser (1983) Adrenergic differentiation of neural crest cells may be dependent upon a neural tube derived factor. *Soc. Neurosci. Abstr.* 9: 897a.
- Iacovitti, L., T. H. Joh, D. H. Park, and R. P. Bunge (1981) Dual expression of neurotransmitter synthesis in cultured autonomic neurons. *J. Neurosci.* 1: 685-690.
- Johnson, M., D. Ross, M. Meyers, R. Rees, R. Bunge, E. Wakshull, and H. Burton (1976) Synaptic vesicle cytochemistry changes when cultured sympathetic neurons develop cholinergic interactions. *Nature* 262: 308-310.
- Kahn, C. R., J. T. Coyle, and A. M. Cohen (1980). Head and trunk cranial crest *in vitro*: Autonomic neuron differentiation. *Dev. Biol.* 77: 340-348.
- Le Douarin, N. M. (1982) *The Neural Crest*, Cambridge University Press, New York.
- Le Douarin, N. M., and M. -A. Teillet (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives of neural crest cells. In *Cell and Tissue Interactions*, J. W. Lash and M. M. Burger, eds., pp. 11-27, Raven Press, New York.
- Le Douarin, N. M., D. Renaud, M. A. Teillet, and G. Le Douarin (1975) Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterospecific transplantations. *Proc. Natl. Acad. Sci. U. S. A.* 72: 728-732.
- Loring, J., B. Glimelius, and J. A. Weston (1982) Extracellular matrix materials influence quail neural crest cell differentiation *in vitro*. *Dev. Biol.* 90: 165-174.
- Maxwell, G. D., P. D. Sietz, and C. E. Rafford (1982) Synthesis and accumulation of putative neurotransmitters by cultured neural crest cells. *J. Neurosci.* 2: 879-888.
- Newgreen, D. F., M. Rittnerman, and E. A. Peters (1979) Morphology and behavior of neural crest cells of chick embryo *in vitro*. *Cell Tissue Res.* 203: 115-140.
- Noden, D. M. (1975) An analysis of the migratory behavior of avian cephalic neural crest cells. *Dev. Biol.* 42: 106-130.
- Norr, S. C. (1973) *In vitro* analysis of sympathetic neuron differentiation from chick neural crest cells. *Dev. Biol.* 34: 16-38.
- Patterson, P. H., and L. L. Y. Chun (1974) The influence of non-neuronal cells on catecholamine and acetylcholine synthesis and accumulation in cultures of dissociated sympathetic neurons. *Proc. Natl. Acad. Sci. U. S. A.* 71: 3607-3610.
- Patterson, P. H., and L. L. Y. Chun (1977a) The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. *Dev. Biol.* 60: 473-481.
- Patterson, P. H., and L. L. Y. Chun (1977b) The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. II. Developmental aspects. *Dev. Biol.* 60: 473-481.
- Potter, D. D., S. C. Landis, and E. J. Furshpen (1981) Adrenergic cholinergic dual-function in cultured sympathetic neurons of the rat. In *Development of the Autonomic Nervous System*, pp. 123-138, CIBA Symposium No. 83.
- Ross, D., M. I. Johnson, and R. P. Bunge (1977) Development of cholinergic

- characteristics in adrenergic neurones is age dependent. *Nature* 267: 536–539.
- Sieber-Blum, M., and C. R. Kahn (1982) Suppression of catecholamine and melanin synthesis and promotion of cholinergic differentiation of quail neural crest cells by heart conditioned medium. *Stem Cells* 2: 344–353.
- Sieber-Blum, M., F. Sieber, and K. M. Yamada (1981) Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells *in vitro*. *Exp. Cell Res.* 133: 285–295.
- Smith, J., M. Fauquet, C. Ziller, and N. M. Le Douarin (1979) Acetylcholine synthesis by mesencephalic neural crest cells in the process of migration *in vivo*. *Nature* 282: 853–855.
- Teillet, M. A., and N. M. Le Douarin (1983) Consequences of neural tube and notochord excision on the development of the peripheral nervous system in the chick embryo. *Dev. Biol.* 98: 192–211.
- Teitelman, G., T. H. John, L. Grayson, D. H. Park, D. J. Reis, and L. Iacovitti (1985) Cholinergic neurons of the chick ciliary ganglia express adrenergic traits *in vivo* and *in vitro*. *J. Neurosci.* 5: 29–39.
- Weston, J. A. (1970) The migration and differentiation of neural crest cells. *Adv. Morphogenesis* 8: 41–114.